IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Ilga Winicov
Serial No.:	09/647,841
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For:	*EXPRESSION OF ALFIN 1 AND METHODS FOR PRODUCING TRANSGENIC PLANTS HAVING INCREASED ROOT GROWTH AND ROOT SPECIFIC GENE ACTIVATION*
Group Art Unit:	1638
Examiner:	Baum, Stuart F.

Declaration of Ilga Winicov

I, Ilga Winicov, do solemnly state as follows:

1. I am a Research Professor in the Department of Plant Biology at Arizona State University, Tempe, Arizona. I have a Ph.D. in Microbiology from the University of Pennsylvania in Philadelphia, Pennsylvania. I have an A.B. in Microbiology from the University of Pennsylvania. I have an M.S. in Bacteriology from the University of Wisconsin at Madison, Wisconsin. My research has focused on gene expression in eukaryotes, especially plant gene regulation in roots and as related to providing acquired salt tolerance in alfalfa and rice.

2. I have reviewed the Office Action mailed September 25, 2003. I am familiar with the paper by Oommen et al (1994, The Plant Cell 6:1789-1803). I have reviewed the abstract by Kagaya corresponding to the publication in 1995, Mol. Gen. Genet. 248:668-674. I will begin my comments with some general remarks on plant promoters.

3. Plant gene promoters have specific sequence elements that respond to different transcription factors which are differentially present in various plant tissues, at different times and in different cells during development and in responses to stress (biotic or abiotic), wounding, infection with fungi, elicitors etc. etc. While negative transcription factors (repressors) are known, most regulation depends on combinations of (positive) transcription factors. It has been shown that roots have a relatively high proportion of root-specific transcription factors compared to flowers/silique specific transcription factors. Constitutively expressed gene promoters have a minimal number of such variable sequence elements and thus are minimally influenced by the

variable cellular conditions when used in transgenic constructs as demonstrated by the Cauliflower mosaic virus (CaMV) 35 S promoter.

4. The Oommen reference discusses the transgenic use of the inducible promoter for Isoflavone Reductase (IFR). Lack of consistent patterns of expression of the alfalfa IFR promoter in tobacco might have been expected from the known plant specificity of the metabolic pathway in which IFR functions. This pathway is found in alfalfa, but not in Solanaceae (tobacco) as stated in paragraph 1 of Oommen's Introduction and Figure 1. The alfalfa IFR promoter would very likely have worked in a consistent manner in chickpea or pea (Leguminosae like alfalfa), from which also IFR transcripts had been isolated. In addition, the IFR gene is highly regulated in both development and stress, where expression can increase by 100 to 1000 fold. Again, this predicts a high level of complexity of the promoter, which was demonstrated by differential expression patterns between the 436 bp and the 765 bp fragments of the promoter.

5. The points made above are further reinforced by "Expression Patterns conferred by Tyrosine/Dihydroxyphenylalanine Decarboxylase Promoters from Opium Poppy are Conserved in Transgenic Tobacco" (Facchini et al. 1998, Plant Physiol. 118:69-81). The authors reported transgenic use in tobacco of promoters from two classes of genes from this multiple gene family in opium poppy. According to the authors, "developmental expression patterns of endogenous genes in opium poppy (Figs. 4 and 5) and tydc promoter-GUS transgenes in tobacco (Figs. 8 and 9 ***are remarkably similar.***" (p. 79, end of right column; emphasis added) In this case the tissue specificity was retained between poppy and tobacco. But, the highly elicitor-induced Tyrosine/Dihydroxyphylalanine Decarboxylase (TYDC1-9) class as represented by *tydc2/7* promoters were broadly expressed in many tissues in both plants. The minimally induced promoter *tydc1* or the only constitutive root specific gene promoters *tydc8* and *tydc9* retained root specificity and minimal or no induction by elicitors.

6. Turning now to the disclosure of the MsPRP2 promoter, it should be noted that the MsPRP2 gene has homologs in widely varied species of plants such as soybean, wheat, maize and pea. Further, Table 2 (pp. 8-10) discloses many different plants in which there are alfin1-responding sequences, which would react similarly to the MsPRP2 promoter, which also responds to alfin1. Like the above examples where the promoted genes were found in a variety

of species and the promoter was effective in a variety of species, the MsPRP2 promoter also can be expected to be active in a variety of plants.

7. In addition, my group's recent work has shown that a heterologous construct of MsPRP2 promoter and the green fluorescent protein resulted in long-lived expression. The following discussion illustrates the preparation and use of constructs containing the MsPRP2 promoter and signal system for heterologous gene expression in plants. FIG. 1 shows the schematic and sequence of this ligation product, which contains a proline residue at the juncture from the primer-introduced XmaI site and allows the MsPRP2 promoter/signal fragment to be used as a cassette for subcloning with other heterologous genes for expression.

8. In summary, the GFP(S65T) sequence (Haseloff et al., 1997, Proc Natl Acad Sci USA, 94(6), 2122-7) was placed under transcriptional control of the -652 to +75 fragment of the MsPRP2 promoter (FIG. 2; SEQ ID NO: 1) or the CaMV 35S promoter. However, since the MsPRP2 (+1/+75 bp) sequence included a putative signal sequence for targeting GFP to the cell wall or secretion (Deutch and Winicov, 1995), we also cloned this putative signal sequence adjacent to GFP in one of the CaMV 35S promoter constructs (top construct of FIG. 1). This permitted us to monitor any effects of the signal sequence on the intracellular accumulation of the transgenically expressed GFP.

9. Constructs were made with a fragment of the MsPRP2 promoter and signal sequence (-652 to +75), where +1 is the A of ATG start of the MsPRP2 coding sequence (Bastola et al, 1998), by PCR using as Forward #1 primer (SEQ ID NO: 2) to introduce an XbaI site at the 5' end and Reverse #1 primer (SEQ ID NO: 3) to introduce an XmaI site at the 3' end of the molecule. All primers are listed in Table 1.

Table 1

Primer	Sequence	SEQ ID NO:
Forward #1	5'GCTCTAGAGGATGCATGATTCGATTAG-3'	2
Reverse #1	5'GGTCCCGGGCAAGCAAGAACAATGAG-3'	3
Forward #2	5'GGACCCGGGGAGTAAAGGAGAAGAACTTTTCAC-3'	4
Reverse #2	5'GGAGATCTGAGCTCTTATTTGTATAGTTC-3'	5
Forward #3	5'GCTCTAGAACACTACACTACTTTCTTTGAACATGAGT	6

	AAAGGAGAAGAACTTTTCAC	
Forward #4	5'GCTCTAGAGTGTATGACTTCATAGTACAC-3'	7

10. The heterologous GFP(S65T) gene was subcloned by PCR introduction of an XmaI site at the 5' end with the Forward #2 primer (SEQ ID NO: 1) and introduction of a BglII site at the 3' end with the Reverse #2 primer SEQ ID NO: 4). The resultant PCR product of GFP(S65T) cDNA encoded the full GFP protein without the methionine at the NH_2 terminus and ended with lysine at the COOH terminus, without any KDEL sequences, which would retain this product in the cytoplasm/endoplasmic reticulum of the transgenic plants. The GFP and MsPRP2 promoter signal fragments were ligated at the XmaI site. FIG. 1 shows the schematic and FIG. 3 gives the sequence of this ligation product (GFP-MsPRPpromsig), which contains a proline residue at the juncture from the primer-introduced XmaI site and allows the MsPRP2 promoter/signal fragment to be used as a cassette for subcloning with other heterologous genes for expression. The GFP-MsPRPpromsig construct was further cloned in the binary vector pGA643 digested with XbaI and BglII (An et al., 1988, Plant Molecular Biology Manual, Vol. Section A (Dordrecht, The Netherlands, Kluwer Academic Publishers)).

11. The efficiency and strength of the MsPRP2 promoter was compared to that of the CaMV 35S promoter, which is one of the most commonly used and strongest promoters for expression of heterologous genes. The GFP(S65T) cDNA subclone was inserted into pGA643 digested with XbaI and Bg1II, adjacent to the 35S promoter of the vector. However, a fragment of the MsPRP2 5' UTR (-28 to +1) was added to the 5' end of the GFP(S65T) sequence by PCR using the Forward #3 primer (SEQ ID NO: 6) in order to provide an efficient plant ribosomal binding site for comparable translation of the two products from the 35S and MsPRP2 promoters. The Forward #3 primer (SEQ ID NO: 6) also introduced the XbaI site necessary for cloning in pGA643 and changes a T to an A in position 24 nucleotides upstream from ATG of the original MsPRP2 sequence. The reverse primer for this PCR reaction with GFP(S65T) was the same as described above for introducing the necessary Bg1II site for cloning in pGA643.

12. Additional control constructs were made to express GFP with the MsPRP2 signal sequence and the 5' UTR under the control of the CaMV 35S promoter in pGA643. This was accomplished introducing an XbaI site at the 5' end of the insert with the Forward #4 primer

(SEQ ID NO: 7), which starts at position 618 in the GFP-MsPRPpromsig construct shown in FIG. 3. All constructs were verified by sequencing.

Agrobacterium transformation of alfalfa and selection of transformants was carried out as described previously (Winicov and Bastola, 1999). Transformation of alfalfa leaf discs from wild type parent (#1) or Alfin1-overexpressing LS-1 plants, callus growth and plant regeneration was carried out as described previously (Winicov and Bastola, 1999). Because LS-1 plants already carry the kanamycin resistance gene, transformant calli-expressing GFP were identified by fluorescence and were initially found to contain both transformed and untransformed cells. However, after three to four months of subculture, most of the GFP-construct-transformed LS-1 lines consisted of 80% or more GFP-expressing cells, possibly due to the double dose of kanamycin resistance gene after the second transformation.

FIGs. 4A-4D show strong, ubiquitous expression of GFP in the roots of alfalfa transformed with the CaMV 35S promoter-MsPRP2-signal-GFP construct with equally strong expression from the MsPRP2promoter-signal-GFP construct as shown in FIGs. 4E-4H.

10. This experimental work proves that the hypothetical example in the specification can function.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that I understand that making willful false statements and the like constitutes conduct punishable by fine or imprisonment or both under Section 1001 of Title 18 of the United States Code, and that any such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Respectfully submitted,

Dated: 6.7.2004

By: _____

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